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EFFECT OF TEMPERATURE ON INTERACTION BETWEEN EGGS AND SPERMATOZOA OF SEA URCHIN

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ABSTRACT

Fertilization in the sea urchin, *Anthocidaris crassispina,* showed marked temperature dependence; high temperatures (15°–30°C) were required for fertilization. In contrast, fertilization in *Hemicentrotus pulcherrimus* occurred over a wide range of temperatures (0°–30°C). The mechanism of this temperature effect in *Anthocidaris* was investigated. The number of sperm bound to the egg surface and the rate of the acrosome reaction were markedly reduced by lower temperatures (0°–10°C). Furthermore, an abnormal elongation of the sperm head tip occurred with higher frequency at lower temperatures. In contrast, the egg activation with calcium ionophore A23187 was not prevented at 10°C. The swimming activity measured by distance traveled was also relatively high at 0°C, although the activity increased as the temperature rose. These results strongly suggest that temperature exerts a direct influence on fertilization in *Anthocidaris* by acting on the acrosome reaction. The increased fertilization rate at higher temperatures in *Anthocidaris* corresponds to the higher temperature observed during the breeding season of this species.

INTRODUCTION

It is well known that development of sea urchin eggs and embryos is influenced by the temperature of the sea water. Generally, embryos develop relatively faster at high temperatures. Most sea urchin species have a specific breeding season. It is interesting that exogastrulation is induced by culture of embryos at temperatures lower than environmental condition during the breeding season (Takahashi *et al.,* 1977). Fujisawa and Amemiya (1979, 1980) also reported that adhesion of dissociated cells in sea urchin embryos is related to the environmental temperature in the breeding season. This is due, to some extent, to energy metabolism which depends on temperature. Glycogen metabolism during early development in the sea urchin, *Hemicentrotus pulcherrimus,* depends upon temperature. It has been reported that glycogen is metabolized at 15°C (Okabayashi and Nakano, 1980) but not at 20°C (Hino and Yasumasu, 1979).

In sea urchin spermatozoa, energy metabolism is influenced by temperature. We already reported (Mita and Yasumasu, 1983) that phospholipid and carbohydrate metabolism in *Hemicentrotus* sperm are activated at 20°C and only glycolysis is carried out at 0°C. Yanagisawa (1967) reported that *Hemicentrotus* sperm maintain a certain ATP level but decrease their phospholipid level after dilution at 14°C. Upon fertilization, a dramatic change in metabolism occurs in both sperm (Fujiwara *et al.,* 1983) and eggs (Yasumasu *et al.,* 1973). However, little is known about the effect of

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temperature on fertilization. In the present study, we compare the effects of temperature on fertilization, using the sea urchins, *Anthocidaris crassispina,* which breeds in summer, and *Hemicentrotus pulcherrimus,* which breeds in winter.

Materials and Methods

Preparation of gametes

Shedding of gametes of the sea urchins, *Anthocidaris* and *Hemicentrotus,* was induced by injection of 0.5 *M* KCl into the coelomic cavity. Semen was collected as "dry sperm" and kept undiluted at 4°C. Eggs were shed directly into sea water. Artificial sea water (ASW) composed of 485 m*M* NaCl, 9.6 m*M* KCl, 10 m*M* $CaCl_2$, 49 m*M* $MgSO_4$, and 10 m*M* Tris-HCl, pH 8.2 was used. The number of eggs and sperm was calculated from the protein concentration as determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. An egg and a spermatozoon contained 100 ng protein and 0.5 pg protein, respectively.

Fertilization in artificial sea water

Eggs were washed 3 times with ASW. The eggs were diluted in ASW (2×10^4 eggs/ml) at a desired temperature and then 5 μl dry sperm (3×10^8 sperm) was added to 1.0 ml egg suspension. Five min after insemination, 0.04 ml of 1% formaldehyde in ASW was dropped into the suspension. Under a light microscope, the percentage of fertilized eggs was calculated by counting the number of eggs with a fertilization membrane.

Measurement of sperm motility

Sperm motility was determined by measuring the distance in which sperm traveled through a glass capillary vessel (d = 1 mm, Vitrex, Modulohm I/S, Denmark) as described by Turner and Giles (1982) with modifications. Dry sperm were diluted in 0.5 ml ASW and a glass capillary vessel prefilled with ASW was immediately inserted into the suspension. The vessel rested at approximately 10°. At the conclusion of the incubation period, the distance which sperm traveled in the vessel was examined under a light microscope.

Oxygen consumption assay

Respiration was measured polarographically using a Clark type oxygen electrode (Yellow Spring Co., U.S.A.). Twenty-five μl of dry sperm ($2.3–2.8 \times 10^9$ sperm) were incubated in 2.5 ml ASW in a closed vessel equipped with an oxygen electrode. Oxygen consumption was determined as described by Robinson and Cooper (1970).

ATP assay

Samples were prepared for ATP assay as described by Mita and Yasumasu (1983). ATP concentration was determined enzymatically as described by Lamprecht and Trantsold (1974).

Treatment of sperm with jelly water

Jelly water was prepared by acidifying the egg suspensions with HCl to pH 5.5 for 4 to 5 min. The eggs were removed by a hand-driven centrifuge, and the supernatant

was adjusted to pH 8.2 with Tris-HCl buffer. Insoluble matter was removed by centrifugation at 12,000 × *g* for 20 min. The concentration of jelly water was estimated by a carbohydrate determination using the method of Dische and Shettles (1951) with fucose as standard. Ten μl of dry sperm ($0.9–1.2 \times 10^9$ sperm) was treated with 1.0 ml of jelly water at a concentration of 20 μg fucose equivalent/ml. After 5 min incubation, the sperm were fixed by adding 1.0 ml of 4% glutaraldehyde in ASW. The percentage of sperm with the acrosome reaction was determined from electron microscopic observation (JEM 100CX, JEOL, and Hitachi HU-11-PS). Samples for scanning electron microscopy were fixed with 2% glutaraldehyde, and washed with ASW. A few drops of concentrated fixed sperm were placed onto a coverslip previously coated with 1% protamin sulfate (Nakarai Chemical Co., Japan). After 10 min, one or two drops of 4% OsO_4 were added to the droplet on the coverslip. The specimens were critical point dried in carbon dioxide and coated with gold (about 250 Å thickness) using an ion coater (IB-2 Eiko Eng'g Co., Japan). Observations were made using a scanning electron microscope (Alpha-10, Akashi Seisaku Co., Japan).

Measurement of adhesion of sperm to egg

Dejellied eggs were diluted in ASW (2×10^4 eggs/ml) at a desired temperature and dry sperm (3×10^8) were added. Vacquier (1979) reported that sperm bound to eggs from 0 to 30 s after addition of sperm to the egg suspension and that at 40 s the sperm began to detach. Therefore, after standing for 40 s the eggs were fixed with 1% glutaraldehyde in ASW. The fixed eggs were photographed under a light microscope and the binding of the sperm to the dejellied eggs was measured by counting the number of sperm bound to the periphery of the egg according to the method of Kato and Sugiyama (1978).

Artificial parthenogenesis by calcium ionophore

Eggs were washed three times with ASW and diluted in the ASW (2×10^4 eggs/ml) at a desired temperature. Calcium ionophore A23187 (Calbiochem Co., U.S.A.) was added to the egg suspension at final concentration of 50 μ*M*. Five min after treatment, 40 μl of 1% formaldehyde in ASW was dropped into the suspension. The number of eggs with fertilization membrane was counted under a light microscope.

Results

Effect of temperature on fertilization

Figure 1 shows the effect of temperature on fertilization based on formation of the fertilization membrane in sea urchin eggs. In *Anthocidaris* which breeds in summer, fertilization did not occur at 5° and 7°C. The fertilization ratio increased between 10° and 15°C, and more than 90% fertilization was obtained at temperatures between 15° and 30°C. On the other hand, fertilization in *Hemicentrotus,* which breeds in winter, did not depend on temperature and fertilization was observed even at 0°C. These data suggest that fertilization in *Anthocidaris* but not *Hemicentrotus* depends on temperature (between 0°–30°C). In order to determine how temperature affects fertilization, therefore, we investigated the effect of temperature on the interaction between eggs and sperm, mainly using *Anthocidaris.*

Effect of temperature on sperm motility

When dry sperm of *Anthocidaris* were diluted in ASW, the ATP level in sperm decreased rapidly within 5 min (Fig. 2). The constant level of ATP was higher at 0°C

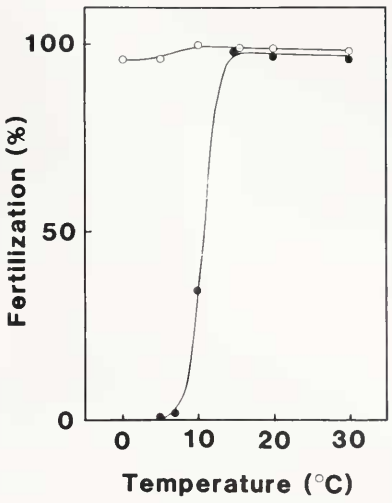

FIGURE 1. Effect of temperature on fertilization of sea urchin eggs. Eggs were inseminated by sperm and the number of eggs that formed a fertilization membrane was calculated. More than 100 eggs were observed. Values represent the mean of three separate experiments. (●): *Anthocidaris crassispina*, (○): *Hemicentrotus pulcherrimus*.

than at 20°C. ATP in sea urchin sperm is produced by phospholipid metabolism (Mohri, 1957; Mita and Yasumasu, 1983) and is consumed by their movement (Gibbons and Gibbons, 1972). Respiration is indispensable for the phospholipid me-

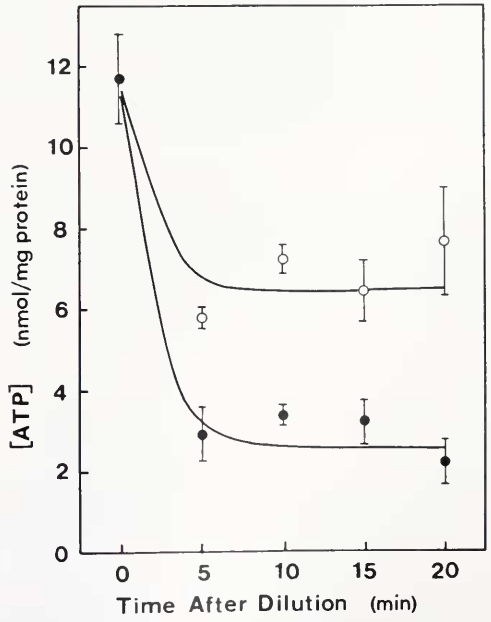

FIGURE 2. Change in the level of ATP after dilution of the dry sperm of the sea urchin, *Anthocidaris crassispina*. Dry sperm were diluted 100 fold in artificial sea water at 0°C (○) and 20°C (●). Values represent the mean of three separate experiments. Vertical bars show S.E.M.

tabolism. The respiratory rate in *Anthocidaris* sperm was correlated with temperature between 5° and 30°C. At low temperature the respiratory rate decreased markedly (Table I). These data suggest that ATP turnover is reduced at low temperatures.

Furthermore, the distance traveled by *Anthocidaris* sperm in a glass capillary vessel was correlated with temperature between 0° and 30°C (Fig. 3). The distance traveled also depends on time after dilution. The distance traveled after 10 min incubation at 20° and 30°C was almost 3 times longer than that at 0°C. However, the *Anthocidaris* sperm were motile even at 0°C. Thus, swimming activity of sperm remains at low temperatures.

Treatment of sperm with jelly water

When sperm of *Anthocidaris* as well as *Hemicentrotus* were treated with jelly water, sperm were agglutinated regardless of temperature between 0° and 30°C. Below 10°C the agglutinated *Anthocidaris* sperm were not dispersed and sperm became immotile, whereas the agglutination in *Hemicentrotus* sperm was released regardless of the temperature between 0° and 30°C. In *Anthocidaris,* the percentage of sperm with reacted acrosome (judging from filament formation) was very low between 5° and 10°C but increased above 15°C (Fig. 4). It is interesting that at low temperatures the acrosome filament is not formed but agglutination is observed.

According to scanning electron microscopic observations, *Anthocidaris* sperm with elongated head tips were more frequently observed at 4°C but they did not have the acrosomal process (Figs. 5c and d). The length of head in the elongated sperm was approximately 0.7–1.0 μm longer than that of the controls (Fig. 5a). The shape of the middle piece of sperm does change during fertilization in several invertebrate species as shown in Figure 5b (Lambert and Epel, 1979; Ikadai and Hoshi, 1981). Change in the shape of the middle piece was also observed among the elongated sperm (Fig. 5c). This may suggest that an incomplete acrosome reaction causes an increase in the number of sperm with elongated head tips at low temperatures. On the other hand, 40 to 60% of the acrosome reactions in *Hemicentrotus* sperm were constantly obtained between 0° and 30°C (Fig. 4).

Binding of sperm to eggs

Figure 6 shows that a number of sperm bound to the periphery of the dejellied eggs of *Anthocidaris* 40 s after addition of dry sperm to egg suspension. The number of bound sperm depends on temperature in the same way that the fertilization ratio was correlated with temperature (Fig. 1). Below 10°C, no spermatozoa bound to

TABLE I

Effect of temperature on oxygen consumption rate of spermatozoa of the sea urchin, Anthocidaris crassispina

Temp. (°C)	Oxygen consumption rate (nmoles O_2/min/mg protein)
5	2.60 ± 0.17
10	17.0 ± 0.52
20	66.9 ± 1.13
30	117.5 ± 0.87

Dry sperm were diluted 100 times in artificial sea water. The value is mean ± S.E.M. obtained in three separate experiments.

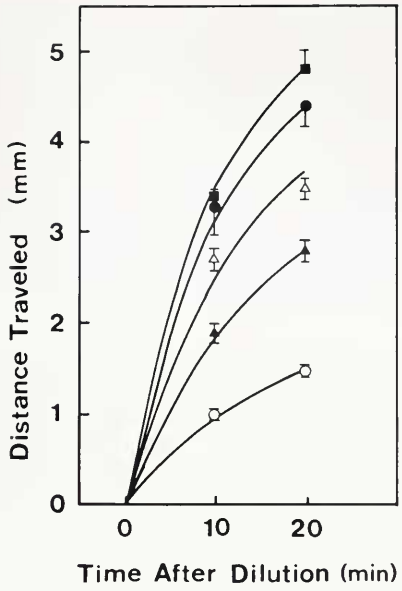

FIGURE 3. Distance traveled by the sperm of the sea urchin, *Anthocidaris crassispina,* after dilution at 0°C (○), 5°C (▲), 10°C (△), 20°C (●), and 30°C (■). Dry sperm were diluted 100 times in artificial sea water. Sperm motility was determined by measurement of the distance which sperm traveled in a glass capillary vessel (d = 1 mm). Each value represents the mean of three separate experiments. Vertical bars show S.E.M.

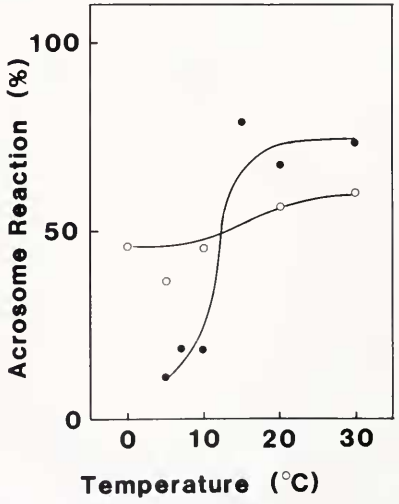

FIGURE 4. Effect of temperature on the acrosome reaction in sea urchin sperm induced by the jelly water. Dry sperm were treated with jelly water. The percentage of the acrosome reaction judging from the filament formation was monitored by electron microscopy. More than 100 sperm were observed. Values represent the mean of three separate experiments. (●): *Anthocidaris crassispina,* (○): *Hemicentrotus pulcherrimus.*

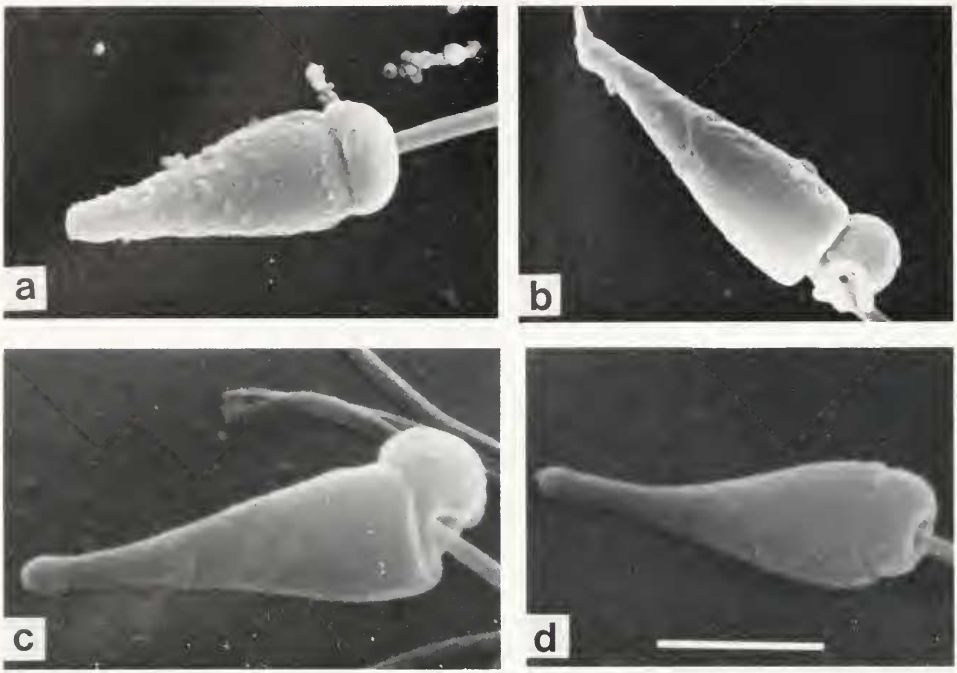

FIGURE 5. The jelly water treatment of the sea urchin sperm, *Anthocidaris crassispina* at low temperature. Dry sperm were treated with the jelly water at 4°C. (a): unreacted sperm, (b): acrosome reacted sperm, and (c) and (d): sperm with elongated head tip. Bar shows 2 μm.

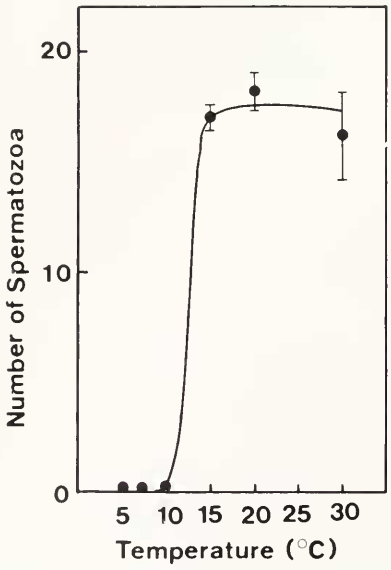

FIGURE 6. Effect of temperature on adhesion of sperm to egg of *Anthocidaris crassispina*. Dry sperm were added to the dejellied egg suspension. The binding of the sperm to the egg was measured by counting the number of sperm bound to the periphery of the egg. Each value represents the mean of five experiments. Vertical bars show S.E.M.

the egg. The number of sperm bound to the egg increased between 10° and 15°C; a constant level of bound sperm was observed above 15°C.

Egg activation with calcium ionophore A23187

Calcium ionophore A23187 activates sea urchin eggs accompanied by formation of the fertilization membrane (Steinhardt and Epel, 1974). About 40% of *Anthocidaris* eggs showed the formation of the fertilization membrane at 4°C, and more than 90% of the eggs were activated at or above 10°C (Fig. 7), after 5 min incubation with the ionophore. The ratio of the fertilization membrane formation did not change at low temperatures, even when eggs were incubated with the ionophore for more than 5 min (data not shown). On the other hand, the activation of *Hemicentrotus* eggs with the ionophore A23187 was not influenced by temperature between 0° and 30°C (Fig. 7) in the same manner that fertilization was not temperature dependent (Fig. 1).

Discussion

The ambient sea water temperature during the breeding season is 0°–17°C for *Hemicentrotus* and 19°–27°C for *Anthocidaris* (Fujisawa and Amemiya, 1979, 1980). This agrees with the results in the present study that successful fertilization of *Anthocidaris* required a temperature higher than 15°C (Fig. 1). It is interesting that fertilization occurs within the range of the environmental temperature at the breeding season. However, *Anthocidaris* eggs could be activated with calcium ionophore A23187 at low temperatures (Fig. 7). This suggests that temperature does not exert a direct influence on activation. Therefore, fertilization of *Anthocidaris* is apparently regulated by the temperature dependency of sperm functions.

Energy metabolism (Fig. 2, Table I) and swimming activity (Fig. 3) in the *Anthocidaris* sperm were correlated with temperature and decreased at low temperatures. The temperature dependency in the swimming activity of sperm may explain the

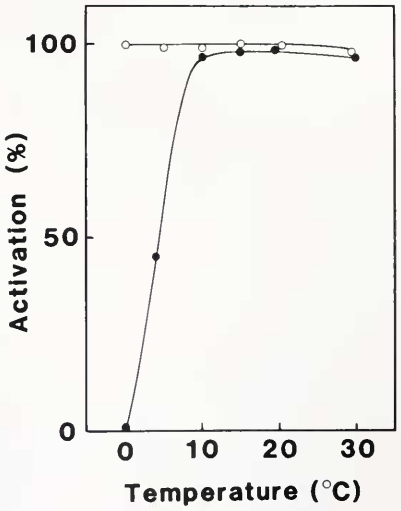

FIGURE 7. Effect of temperature on sea urchin egg activation by calcium ionophore A23187. Eggs were activated by the ionophore and the percentage of eggs with fertilization membrane was calculated. More than 100 eggs were observed. Each value represents the mean of three separate experiments. (●): *Anthocidaris crassispina*, (○): *Hemicentrotus pulcherrimus*.

failure of fertilization at low temperatures. The number of sperm bound to eggs in *Anthocidaris* was almost zero at temperatures lower than 10°C but was remarkably increased above 15°C (Fig. 6). Before adhesion of egg and spermatozoa, sea urchin sperm undergo the acrosome reaction (Dan, 1952; Afzelius and Murray, 1957). The percentage of the acrosome-reacted sperm in *Anthocidaris* also increased above 15°C (Fig. 4). These data suggest that the temperature at which the acrosome reaction occurs is closely related to successful fertilization. On the other hand, the acrosome reaction in *Hemicentrotus* sperm was not influenced by temperatures between 0° and 30°C (Fig. 4). Thus, fertilization in *Hemicentrotus* occurred at a wide range of temperatures from 0° to 30°C (Fig. 1).

There may be three reasons why the acrosome reaction in *Anthocidaris* does not occur frequently at low temperatures. First, activity of an acrosin-like enzyme which contributes to the acrosome reaction (Levine *et al.*, 1978) may depend on temperature in *Anthocidaris* but may not be influenced by temperature in *Hemicentrotus*. Second, the cell membrane of *Hemicentrotus* sperm may be more fluid at low temperatures than that of *Anthocidaris* sperm, suggesting that a low temperature condition prevents the acrosome from undergoing exocytosis. Finally, it is also possible that the polymerization of actin in the acrosomal rod is reduced under a low temperature condition. Unfortunately, little is known about these phenomena. In the present study we have reported that sperm with elongated head tips were observed at low temperature (Figs. 5c and d). The occurrence of the sperm with an elongated head tip may induce the failure of fertilization in *Anthocidaris* at low temperatures.

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